FAST AND ENZYMELESS CLONING OF NUCLEIC ACID FRAGMENTS

Background of the Invention

Field of the Invention

The present invention relates to methods, systems and kits for fast and enzymeless cloning of nucleic acid fragments into vectors and for forced cloning selection for successful transformation.

Description of the Related Art

Traditional molecular cloning involves the use of recombinant DNA technology to propagate DNA fragments inside a foreign host. Generally, the DNA fragments are isolated from cDNA libraries or chromosomes and subcloned into a vector utilizing various enzymes. The DNA fragment-containing vector is introduced into a host cell according to various methods of transformation. A selection marker is usually included in the vector to increase the probability that the host cell has the DNA fragment-containing vector. Following introduction into the host cell and selection of the host cell containing the vector, the DNA fragment within the vector can then be replicated along with the host cell DNA. The DNA fragment-containing vector then can be isolated and purified from the host cell and transfected into animal cells or tissues for functional analysis of the encoded gene product.

Although the traditional enzymatic cloning methods have advantages such as pinpoint accuracy, they also have significant drawbacks. As mentioned, the methods require the use various enzymes that can be very expensive. In addition, the same DNA fragment has to be enzymatically treated every time it is introduced into a different vector. Also, the methods involve slow and laborious processes. Selection of host cells containing the DNA fragment-containing vector entails significant labor and is still an uncertain process. Traditional cloning methods, even in conjunction with the use of polymerase chain reaction (PCR), are still time consuming, costly and difficult to automate.

The present invention provides simple and rapid methods, systems and kits for cloning nucleic acid fragments.

Summary of the Invention

In one embodiment, the present invention relates to a method for cloning a nucleic acid fragment into a vector by flanking the fragment with first and second adapter sequences. The fragment can be contacted with the vector having sequences homologous to the first and second adapter sequences under conditions such that the nucleic acid fragment is incorporated into the vector by homologous recombination *in vivo* in a host cell.

The nucleic acid fragment can be generated by polymerase chain reaction (PCR). The first and second adapter sequences can be incorporated to the nucleic acid fragment by PCR. The resulting nucleic acid fragment can be a transcriptionally active PCR fragment.

The first and second adapter sequences further can include a functional element. The functional element can include a promoter, a terminator, a nucleic acid fragment encoding a selection marker gene, a nucleic acid fragment encoding a known protein, a fusion tag, a nucleic acid fragment encoding a portion of a selection marker gene, a nucleic acid fragment encoding a growth promoting protein, a nucleic acid fragment encoding a transcription factor, a nucleic acid fragment encoding an autofluorescent protein (e.g. GFP), and the like.

The nucleic acid fragment can include an additional element. The additional element can be, for example, an operably linked promoter, a termination sequence, an operon, a fusion tag, a signal peptide for intracellular or intercellular trafficking, a peptide, a protein, an antisense sequence, a ribozyme, a protein binding site, and the like.

The promoter can be, for example, a promoter from a plant or a plant pathogen, such as cauliflower mosaic virus, from a mammal or a mammalian pathogen, such as CMV, SV40, MMV, HIV, from a fungus, such as yeast (Gal 4 promoter), from a bacterium or a bacterial phage, for example, lambda, T3, T7, SP6 and the like. The terminator sequence can be derived from a plant, a procaryotyic source or a eukaryotic

source, such as SV40, bovine growth hormone, rabbit beta-globin i, and the like. The operon can be, for example, lac operon, Tet/on operon, Tet/off operon, trp operon. The fusion tag can include 6x or 8x his-tag, GST tag, fluorescent protein tag, Flag tag, HA tag, and the like. The protein can include an enzyme, a receptor, a transcription factor, a lymphokine, a hormone, an antigen, and the like.

The vector can be, for example a plasmid, a cosmid, a bacterial artificial chromasome (BAC), and the like. The plasmid can be CoE1, PR100, R2, pACYC, and the like. The vector can also include a functional selection marker. The functional selection marker can be, for example, a resistance gene for kanamycin, amplicillin, blasticidin, carbonicillin, tetracycline, chloramphenicol, and the like. The vector further can include a dysfunctional selection marker that lacks a critical element, and wherein the critical element is supplied by said nucleic acid fragment upon successful homologous recombination. The dysfunctional selection marker can be, for example, kenamycin resistance gene, kanamycin resistance gene, ampicillin resistance gene, blasticidin resistance gene, carbonicillin resistance gene, tetracycline resistance gene, chloramphenicol resistance gene, and the like. Further, the dysfunctional selection marker can be, for example, a reporter gene, such as the lacZ gene, and the like.

The vector can include a negative selection element detrimental to host cell growth. The negative selection element can be disabled by said nucleic acid fragment upon successful homologous recombination. The negative selection element can be inducible. The negative selection element can be, for example, a mouse GATA-1 gene. The vector can include a dysfunctional selection marker and a negative selection element.

The host cell can be a bacterium. The bacterium can be capable of *in vivo* recombination. Examples of a bacterium include JC8679, TB1, DH5α, DH5, HB101, JM101, JM109, LE392, and the like. The plasmid can be maintained in the host cell under the selection condition selecting for the functional selection marker.

The first and second adapter sequences can be at least 11 bp. The first and second adapter sequences can be at least 40 bp. The first and second adapter sequences can be at least 50 bp. The first and second adapter sequences can be at least 60 bp. The first and second adapter sequences can be greater than 60 bp.

The contacting can include transforming a host cell with the vector and the nucleic acid fragment. The transformation can include electroporation, chemical transformation, and the like.

The host cell can be a bacterium bearing the vector. The bacterium can be capable of *in vivo* recombination. The bacterium can be, for example, JC8679, TB1, DH5α, DH5, HB101, JM101, JM109, LE392, and the like. The contacting of the vector and the nucleic acid fragment can include transforming the host cell bearing the vector with the nucleic acid fragment.

The vector can be a plasmid. The plasmid can include a functional selection marker. The functional selection marker can be, for example, a resistance gene for kanamycin, ampicillin, blasticidin, carbonicillin, tetracycline, chloramphenicol, and the like. The plasmid can include a dysfunctional selection marker that lacks a critical element, and wherein the critical element is supplied by said nucleic acid fragment upon successful homologous recombination. The dysfunctional selection marker can be, for example a resistance gene for kanamycin, kenamycin, ampicilin, blasticidin, carbonicillin, tetracycline, chloramphenicol, and the like. Further, the dysfunctional selection marker can be, for example, a reporter gene, such as the lacZ gene, and the like.

The vector can include a negative selection element detrimental to host cell growth, and the negative selection element can be disabled by the nucleic acid fragment upon successful homologous recombination. The negative selection element can be inducible, for example. The negative selection element can be, for example GATA-1 gene. The vector can include a dysfunctional selection marker and a negative selection element.

One embodiment of the present invention relates to a method for selecting for successful transformation of a vector by a nucleic acid insert. The method can provide a nucleic acid insert flanked by first and second adapter sequences that is adapted for recombining with homologous sequences in a vector. The vector can have a dysfunctional selection marker lacking a critical element and the nucleic acid insert contains the critical element. The nucleic acid insert can be contacted with the vector to effect recombination at homologous sites such that the critical element is supplied to the

vector by the nucleic acid insert and the dysfunctional selection marker is restored to a functional one. The successfully restored selection marker can be selected for based upon growth of a host containing the successfully recombined vector that allows the host to grow or be identified in a selective environment. The recombining can be by homologous recombination.

A further embodiment of the present invention relates to a method for selecting for successful transformation of a vector by a nucleic acid insert. The method can include providing a nucleic acid insert flanked by first and second adapter sequences that is adapted for recombining with homologous sequences in a vector. The vector can include a negative selection element detrimental to cell growth. The nucleic acid insert can be contacted with the nucleic acid insert to effect recombination at homologous sites such that the negative selection element is disabled. Successful transformation can be selected for based on the absence of a functional negative selection element. The negative selection element can be inducible, for example. The selection step can include inducing the negative selection element. The embodied method utilizing the negative selection element further can include the method for selecting for successful transformation of a vector by a nucleic acid insert wherein the vector includes a dysfunctional selection marker lacking a critical element and the nucleic acid insert contains the critical element, as discussed above. The negative selection element can be disabled by insertion of a sequence encoding a selection marker.

Another embodiment of the present invention relates to a system for cloning a nucleic acid fragment into a vector without restriction enzyme, ligase, gyrase, single stranded DNA binding protein, or other DNA modifying enzymes. The system can include a nucleic acid fragment flanked by first and second adapter sequences and a vector having sequences homologous to the first and second adapter sequences wherein the nucleic acid fragment is adapted to incorporate into the vector by homologous recombination.

The nucleic acid fragment flanked by the first and the second adapter sequences can be generated by PCR without the use of a restriction enzyme, a ligase, a gyrase, a single stranded DNA binding protein, or any other DNA modifying enzyme. The

nucleic acid fragment flanked by the first and the second adapter sequences can be a transcriptionally active PCR fragment.

A further embodiment relates to a system for cloning a nucleic acid fragment into a bacterium without the use of a restriction enzyme, a ligase, a gyrase, a single stranded DNA binding protein, or any other DNA modifying enzyme. The system can include a nucleic acid fragment flanked by first and second adapter sequences and a bacterium bearing a vector, the vector having sequences homologous to the first and second adapter sequences, wherein the nucleic acid fragment is adapted to incorporate into the vector within the bacterium by homologous recombination.

In still another embodiment, the present invention relates to a kit for cloning a nucleic acid fragment into a vector. The system can include reagents for amplification of the nucleic acid fragment, wherein the reagents upon amplification can provide for a nucleic acid fragment flanked by first and second adapter sequences, a vector, a competent cell, or a competent cell bearing the vector, and the like. The competent cell can be ready to be transformed by electroporation, chemical transformation, and the like. The competent cell or the competent cell bearing the vector can be a bacterium. The bacterium can be capable of *in vivo* recombination.

The various specific features discussed above also can be used in the other embodiments discussed below.

Brief Description of the Drawings

Figure 1 depicts an embodiment of the present invention related to fast and enzymeless cloning into a vector.

Figure 2 illustrates one embodiment related to generating and cloning a nucleic acid fragment into a vector.

Figure 3 depicts an exemplary receptor plasmid vector.

Figure 4 illustrates one embodiment relating to selection of a successfully transformed host.

Detailed Description of the Invention

The present invention, in at least certain embodiments, overcomes many of the above-described drawbacks of traditional cloning. The present invention includes methods, systems and kits for fast and simple molecular cloning of a nucleic acid fragment directly into a vector without the use of enzymes, such as restriction endonuclease, DNA ligase or any other DNA modifying enzyme. The present invention also includes simple and fast methods for selecting host cells containing the desired DNA fragment and vector.

The present invention generally provides methods, systems and kits for cloning a nucleic acid fragment into a vector by homologous recombination within a host cell. When the common sequences on both the 5' and 3' ends of the nucleic acid fragment are complimentary with terminal sequences in a linearized empty vector, and the fragment and linearized vector are introduced, by electroporation, for example, together into a host cell, they recombine resulting in a new expression vector with the fragment directionally inserted. In alternative embodiments the host cell can include the linearized empty vector so that only the nucleic acid fragment is introduced into the host cell. It should be noted that in alternative embodiments of the present invention the vector can be circularized, and as used herein a vector can be either linearized or circular. The host cell is converted into an expression vector through homologous recombination. In principle this approach can be applied generally as an alternative to conventional cloning methods.

One embodiment of the present invention includes a method for cloning a nucleic acid fragment flanked by first and second adapter sequences into a vector having homologous first and second adapter sequences. The nucleic acid fragment incorporates into the vector by homologous recombination within a host cell.

More specifically, referring now to the embodiment of the present invention depicted in Fig. **1**, a nucleic acid fragment **10** is flanked by a first adapter sequence **12** and a second adapter sequence **14**. A vector **16** also has a first vector adapter sequence **18** and a second vector adapter sequence **20**, which sequences are respectively homologous to the first and second adapter sequences **12, 14** of the nucleic acid fragment **10**.

In embodiments of the present invention the homologous first and second adapter sequences **12, 14** can be at least 11 bp. In other embodiments the homologous first and second adapter sequences can be at least 15 or 20 bp. Further, in embodiments the homologous first and second adapter sequences can be at least 25 or 30 bp. The homologous first and second adapter sequences can be at least 40 bp. Also, the homologous first and second adapter sequences can be at least 50 bp. In preferred embodiments the homologous first and second adapter sequences are at least 60 bp. In more preferred embodiments the homologous first and second adapter sequences are at greater than 60 bp.

The nucleic acid fragment **10** can also include a coding region **26** for a sequence or gene of interest. As used herein coding region refers generically to a region of the nucleic acid fragment **10** that can encode, for example, an operably linked promoter, a termination sequence, an operon, a fusion tag, a signal peptide for intracellular or intercellular trafficking, a peptide, a protein, an antisense sequence, a ribozyme, a protein binding site, and the like.

In one embodiment the coding region **26** can encode a protein. The protein can include enzymes receptors, transcription factors, lymphokines, hormones, antigens. In one embodiment the coding region **26** can include a gene encoding a product which is absent or present at reduced levels in an organism. Nonlimiting examples of these gene products are the cystic fibrosis transmembrane regulator (CFTR), insulin, dystrophin, interleukin-2, interleukin-12, erythropoietin, gamma interferon, and granulocyte macrophage colony stimulating factor (GM-CSF). As noted above, one of skill in the art need only know the terminal sequences of the coding region gene of interest in order to generate a nucleic acid fragment **10** from a natural source or library comprising the gene with the first and second adapter sequences **12, 14**.

The nucleic acid fragment **10** having first and second adapter sequences **12, 14** can be generated by methods well known to those of skill in the art. Referring to the embodiment of the invention depicted in Fig. **2**, a gene of interest **26** with known 5' and 3' sequences undergoes PCR along with overlapping 5' and 3' priming oligonucleotides **30, 32**. The priming oligonucleotides can be obtained by methods known in the art, including manufacture by commercial suppliers. A primary fragment **34** with adapter

sequences is generated. The adapter sequences flanking the gene of interest can be homologous to sequences on a vector or to sequences from other 5' or 3' fragments to be used in a subsequent PCR, as will be discussed more fully below. The method depicted in Fig. **2** is more fully described in U.S. Patent Application No. 09/535,262, "Methods for Generating Transcriptionally Active DNA Fragments," which is hereby incorporated by reference in its entirety.

The nucleic acid fragment depicted in Fig. **1** can also include a functional element. In one embodiment the first and second adapter sequences **12, 14** can include the functional element. Fig. **2** illustrates one method for generating a nucleic acid fragment with functional elements. The primary fragment generated, as discussed above, has flanking sequences homologous to sequences on a 5' fragment **36** and a 3' fragment **40**, respectively. The 5' and 3' fragments **36, 40** include functional elements **46** and **50**, as well as a first and a second adapter sequence homologous to sequences on the primary fragment **34**. A 5' primer **42** and 3' primer **44** for PCR can also be included. All undergo PCR. The resulting fragment **52** has a new 5' element **54** and a new 3' element **56** that include a functional element and terminal flanking sequences homologous to sequences on a vector. As noted above, the method is more fully described in U.S. Patent Application No. 09/535,262, "Methods for Generating Transcriptionally Active DNA Fragments," Liang, et al, which is hereby incorporated by reference in its entirety. For purposes of the present invention "transcriptionally active PCR fragment" or "transcriptionally active DNA fragment" refers to a nucleic acid fragment having a promoter and terminator sequence included therewith such that the fragment can be transcribed within a host cell. Depending upon the adapter sequences, the resulting vectors are useful for a variety of different applications.

One of skill in the art can readily configure orientations and generate nucleic acid fragments with such functional elements by methods well known in the art. In some embodiments, for example, the functional element can be a promoter, a terminator, a nucleic acid fragment encoding a selection marker gene, a nucleic acid fragment encoding a known protein, such as a fusion tag, a nucleic acid fragment encoding a portion of a selection marker gene, a nucleic acid fragment encoding a

growth promoting protein, a nucleic acid fragment encoding a transcription factor, a nucleic acid fragment encoding an autofluorescent protein (e.g. GFP), and the like.

Nucleic acid fragments flanked by adapter sequences suitable for the purposes of the present invention can be generated using the TAP Express™ system (Gene Therapy Systems, San Diego, CA). The TAP Express™ uses nested PCR to append adapter sequences, which can include additional sequences such as a promoter and a terminator sequence, onto PCR fragments so that they become transcriptionally active and can be used directly in *in vitro* and *in vivo* transfection experiments. The TAP Express™ system can be used to generate a large numbers of genes that can be conveniently amplified and introduced into functional assays in a single day, a task that is impractical or impossible using conventional cloning methodology.

As used herein, the term "promoter" is a DNA sequence which extends upstream from the transcription initiation site and is involved in binding of RNA polymerase. The promoter may contain several short (<10 base pair) sequence elements that bind transcription factors, generally dispersed over >200 base pairs. A promoter that contains only elements recognized by general and upstream factors is usually transcribed in any cell type. Such promoters may be responsible for expression of cellular genes that are constitutively expressed (sometimes called housekeeping genes). There are also tissue-specific promoters limited to particular cell types, such as the human metallothionein (MT) promoter that is upregulated by heavy metal ions and glucocorticoids. The promoter can be selected based upon consideration of the desired use for the nucleic acid fragment. One skilled in the art easily can select an appropriate promoter according the uses of the nucleic acid fragment. For example, if the nucleic acid sequence encodes a gene with potential utility in human cells, then a promoter capable of promoting transcription in mammalian cells can be selected. Other examples of a promoter includes a promoter from a plant or a plant pathogen, such as cauliflower mosaic virus, and the like. The promoter can be from a mammal or a mammalian pathogen such as CMV, SV40, MMV, HIV, and the like. In other examples the promoter can be from a fungus such as a yeast (Gal 4 promoter), and the like, while in other examples it can be from bacteria or bacterial phage, for example lambda, T3, T7, SP6, and the like.

As used herein, the term "terminator" is a DNA sequence represented at the end of the transcript that causes RNA polymerase to terminate transcription. This occurs at a discrete site downstream of the mature 3' end, which is generated by cleavage and polyadenylation. For example, the terminator sequence can be derived from a plant, a procaryotyic or a eukaryotic source, such as SV40, bovine growth hormone, rabbit beta-globin i, and the like.

As used herein, the term "operon" is a controllable unit of transcription consisting of a number of structural genes transcribed together. An operon can contain at least two distinct regions, the operator and the promoter. Examples of operons include the lac operon, Tet/on operon, Tet/off operon, trp operon, and the like.

Term "fusion tag" is used herein to refer generally to a nucleic acid sequence encoding a molecule used to quantify, capture, purify, visualize, etc., the expressed protein to which the fusion tag is fused or attached. Examples of fusion tags include 6x or 8x his-tag, GST tag, fluorescent protein tag, Flag tag, HA tag, and the like.

In one embodiment the nucleic acid fragment **10** and the vector **16** are introduced together into a host cell **24**. Within the host cell **24** the nucleic acid fragment **10** incorporates into the vector **16** by *in vivo* homologous recombination. The homologous sequence between the nucleic acid fragment and the vector can be recognized by the DNA recombination and repair mechanism (e.g., in *E. coli*) and joined together.

In another embodiment, the vector **16** first can be mixed with the competent host cell **24** and frozen away immediately. The competent host cell/vector mixture can be aliquotted and kept frozen. The transformation can be performed upon thawing the aliquot and adding only the desired nucleic acid fragment **10** or PCR product.

In another embodiment the nucleic acid fragment **10** is introduced into the host cell **24** bearing the vector **16**. For example, the vector **16** may be replicated with the host cell. Once the nucleic acid fragment **10** is introduced into the host cell **24** bearing the vector **16**, it incorporates into the vector by *in vivo* by homologous recombination.

The vector **16** can be a plasmid, a cosmid, a bacterial artificial chromasome (BAC), or the like. Examples of a plasmid include CoE1, PR100, R2, pACYC, and the like. Fig. **3** depicts one example of a plasmid that can be used in the present invention.

The plasmid can include a functional or intact selection marker for growth. For example, Fig. **3** illustrates a plasmid vector **60** that includes an intact selection marker **62** for growth. Examples of a functional selection marker include a resistance gene for kanamycin, kenamycin, ampicillin, blasticidin, carbonicillin, tetracycline, chloramphenicol, and the like. The vector can be maintained in the host cell under the selection condition selecting for the functional selection marker.

Further, embodiments of the present invention include methods for selecting for successful transformation of a vector by a nucleic acid fragment. In one embodiment of the present invention the vector can include a dysfunctional selection marker that lacks a critical element. Upon successful *in vivo* homologous recombination, the lacked critical element is supplied to the vector by the nucleic acid fragment. As the homologous recombination and repair mechanism is not yet well characterized, it is of a relatively low frequency, making the identification of the intended recombined vector difficult. The inclusion of a critical element necessary for the viability of the host cell will facilitate selection of the intended vector, because only the correctly recombined vector can survive with the host, while a host only carrying either the insert or the vector alone cannot survive. This embodiment of the present invention can be referred to as "forced cloning." As an example, Fig. **3** illustrates a plasmid vector **60** having a dysfunctional or crippled marker **64** for recombination selection. Examples of a dysfunctional selection marker include an incomplete sequence of a resistance gene, for example kanamycin, kenamycin, ampicillin, blasticidin, carbonicillin, tetracycline, chloramphenicol, and the like. Additional examples include reporter genes, such as the lacZ gene, and the like. As used herein reporter gene refers to a gene that is used to locate or identify another gene. Other dysfunctional selection markers can include genes encoding products necessary for a metabolic or cellular pathway, and the like. One of skill in the art can easily select other useful "dysfunctional" selection markers based upon knowledge and skill common in the art.

The incomplete sequence, lacking a critical element, is completed by insertion of the lacked sequence or critical element upon a successful homologous recombination. In some embodiments the incomplete sequence can be missing at least a portion of a protein coding region, or, e.g., all or part of a regulatory element such as a promoter or

termination sequence. The missing portion can be a major portion of the critical element or selection marker, or only a minor portion (e.g., one or more critical nucleotide residues).

A successfully transformed host can also be selecting for by a negative selection method. The vector can include a negative selection element. A negative selection element can be a sequence that encodes a molecule that is detrimental to growth of the host cell, such as, for example, the mouse GATA-1 gene product which is toxic to some cells. The toxic gene can be interrupted or replaced by a nucleic acid sequence that is correctly incorporated into the vector by homologous recombination. Only cells with a successfully incorporated "interrupting" sequence survive, because only those cells lack the toxic gene product. The negative selection element can also encode for molecules that block cell metabolism or prevent efficient transcription, and the like. One of skill in the art can easily select other elements that will work with the present invention.

In one embodiment of the present invention the host cell can be a bacterium. In preferred embodiments the bacterium is capable of *in vivo* recombination. Examples of a bacterium include JC8679, TB1, DH5α, DH5, HB101, JM101, JM109, LE392, and the like.

In one embodiment the host cell can bear the vector. In preferred embodiments the host cell can be a bacterium. In more preferred embodiments the bacterium is capable of *in vivo* recombination. Examples of a bacterium that can bear the vector, as described above, can include JC8679, TB1, DH5α, DH5, HB101, JM101, JM109, LE392, and the like. In embodiments where the host cell bears the vector, only the nucleic acid fragment is introduced into the host cell, for example, by electroporation or chemical transformation.

As mentioned above, the nucleic acid fragment and the vector can be introduced together into the host cell. Alternatively, the vector first can be introduced into the cell followed by a later introduction of the nucleic acid fragment or simply the nucleic acid fragment can be introduced into the host cell in order to transform the cell. Further, the host cell can include a vector that replicates with the cell, thus obviating the need to introduce a vector into the host cell. In preferred embodiments the nucleic acid fragment and/or vector can be introduced by electroporation, chemical transformation,

and the like. In one preferred embodiment the nucleic acid fragment and the vector are introduced into an *E. Coli* cell by high efficiency electroporation. For example, in "high efficiency electroporation," as used herein, each microgram of a supercoiled plasmid, when delivered into a cell (such as *E. Coli*, for example) by electroporation, would be able to produce 10^{10} or more colonies.

In another embodiment the present invention also includes high efficiency electroporation-competent cells. These cells significantly facilitate the introduction of insert and vector into the host cells, thus improving the efficiency of recombination, which is a bi-molecule reaction, that is exponentially dependent on the amount of substrate (the fragment and vector). Examples of these cells include JC8679, TB1, HB101, and the like.

As discussed briefly above, the present invention also includes methods and systems for forced cloning. Traditionally, a cloning vector will include a selection marker, such as a resistance gene, so that only a host cell having a properly incorporated DNA insert and vector will grow in a selective medium. However, the host cell may incorporate a vector having the resistance gene without the desired insert or with only a portion of the insert. Thus, host cell colonies will have to be screened, potentially at a significant time, material and labor cost, in order to identify a colony having the proper vector and insert.

Embodiments of the present invention relate to methods for selecting for the successful transformation of a vector by a nucleic acid insert. Referring to Fig. **1**, in one embodiment the vector **16** is prepared with a dysfunctional selection marker that lacks a critical element. The nucleic acid fragment **10** can include the critical element. As used herein, the term "critical element" can refer to any sequence on the nucleic acid fragment **10** that, upon incorporation with the vector **16**, restores functionality to a selection marker. For example, the critical element can be a promoter, a terminator, a nucleic acid fragment encoding a selection marker gene, a nucleic acid fragment encoding a known protein such as fusion tag, a nucleic acid fragment encoding a portion of a selection marker gene, a nucleic acid fragment encoding a growth promoting protein, a nucleic acid fragment encoding a transcription factor, a nucleic acid fragment encoding an autofluorescent protein (e.g. GFP), and the like. The resulting vector

within the transformed host cell allows the host cell to grow in a selective medium. Thus, only host cells that are properly transformed with vector and nucleic acid fragment will grow. These embodiments minimize the need for subsequent, labor intensive and time consuming identification and selection of transformed cells.

In one embodiment, the vector can have a dysfunctional antibiotic resistance gene. For example, the vector can be prepared having an interrupted antibiotic resistance gene. The nucleic acid fragment is engineered to restore the functional antibiotic resistance gene upon incorporation into the vector by homologous recombination. The host cell having the "restored" vector can then be plated in a selective growth media. Any host cell lacking the "restored" vector will be unable to grow in the selective media.

In addition to the embodiments described above related to positive selection, embodiments of the present invention include methods, systems and kits relating to negative selection for a successful transformant. In one such embodiment, the vector can have a negative selection element that is detrimental to cell growth. For example, the negative selection element can be sequence that encodes a molecule that is toxic to the cell, a molecule that stops or prevents transcription, a molecule that is otherwise detrimental to growth of the host cell, and the like. When a nucleic acid fragment incorporates with the vector by homologous recombination within the host cell, the negative selection element is disabled. Disabling the negative selection element allows the host cell to grow, thus only cells with proper insertion of the nucleic acid fragment into the vector will survive and be selected.

The negative selection element can be inducible. For example, the vector can have a functional suicide gene or other negative selection element. The suicide gene can be replaced or disabled upon incorporation of the nucleic acid fragment into the vector by homologous recombination.

For example, referring to Fig. **4**, a vector **76** can be prepared having a negative selection element **80**, in this embodiment a mouse GATA-1 transcription factor gene. The negative selection element can be inserted between the first adapter sequence **82** and the second adapter sequence **84**. The first and second adapter sequences **82**, **84** have regions homologous to the ends of a nucleic acid fragment **68** that is to be cloned.

The nucleic acid fragment **68** can be generated by PCR or any other suitable method, as discussed herein. The nucleic acid fragment **68** can encode some gene of interest **70** as discussed above. The fragment **68** includes a first adapter sequence **72** and a second adapter sequence **74**, which are homologous to the first and second adapter sequences **82, 84** on the vector **76**. The first and second adapter sequences **72, 74**, as discussed above can also include additional elements, such as sequences encoding a promoter, a terminator, an operon, a fusion tag, and the like.

The negative selection element **80**, in this case the GATA-1 gene, is under the control of TAC promoter inducible by IPTG, and its product is able to bind to the bacterial origin of replication, therefore resulting in a rapid arrest of cell growth. The nucleic acid fragment **68** upon incorporation into the vector **76** by homologous recombination will replace the negative selection element **80**, thus enabling the host cell to grow in a selective media. Any host cells lacking the recombined vector will be unable to grow in the selective media.

The negative selection methods and systems can be combined with the other systems, methods, and kits, including for example, forced cloning. The nucleic acid insert can encode a critical element, as described above, that restores function to a disabled selection marker, while at the same time disabling a negative selection element, such as a suicide gene. Alternatively, the forced cloning methods, systems and kits can be used independently, in conjunction with the negative selection methods, systems and kits. In one embodiment, two nucleic acid fragments may be introduced into the host cell.

Another embodiment of the present invention relates to a system for cloning a nucleic acid fragment into a vector without restriction enzyme, ligase, gyrase, single stranded DNA binding protein, or other DNA modifying enzymes. The system can include a nucleic acid fragment flanked by first and second adapter sequences, and a vector having sequences homologous to the first and second adapter sequences. The nucleic acid fragment is adapted to incorporate into the vector by homologous recombination.

The nucleic acid fragment flanked by the first and the second adapter sequences can be generated by PCR without the use of a restriction enzyme, a ligase, a gyrase, a

single stranded DNA binding protein, or any other DNA modifying enzyme as discussed above or according to any other method known in the art. The nucleic acid fragment flanked by the first and the second adapter sequences can be a transcriptionally active PCR fragment.

One embodiment of the present invention relates to a system for cloning a nucleic acid fragment into a bacterium bearing a vector, without the use of a restriction enzyme, a ligase, a gyrase, a single stranded DNA binding protein, or any other DNA modifying enzyme. The system can include a nucleic acid fragment flanked by first and second adapter sequences and a bacterium bearing a vector having sequences homologous to the first and second adapter sequences. The nucleic acid fragment is adapted to incorporate into the vector within the bacterium by homologous recombination.

A further embodiment relates to a kit for cloning a nucleic acid fragment into a vector. The kit can include reagents for amplification of the nucleic acid fragment. Suitable reagents may include, for example, TAQ polymerase and/or PCR reagents such as adapter sequences capable of acting as primers for nested PCR and including regions of homology to a nucleic fragment of interest and regions added onto the ends of the nucleic acid fragment of interest upon successful amplification steps, as explained in more detail in U.S. Application No. 09/535,262, discussed above. The reagents upon amplification can provide for a nucleic acid fragment flanked by first and second adapter sequences, a vector, a competent cell, or a competent cell bearing the vector. The competent cell can form a part of the kit and can be ready to be transformed by electroporation, chemical transformation, or any like method known in the art. In preferred embodiments, the competent cell or the competent cell bearing the vector is bacteria. In other preferred embodiments the bacteria can be capable of *in vivo* recombination.

EXAMPLES

EXAMPLE 1

Generation of transcriptionally active PCR fragment encoding Chloramphenicol acetyltransferase (CAT)

The following components were combined in a 50 µl polymerase chain reaction (PCR): primers; 5'CTGCAGGCACCGTCGTCGACTTAACAATGGAGAAAAAAATCACTGG3'; and 5'CATCAATGTATCTTATCATGTCTGATTACGCCCCGCCCTGCCACTC3', 1 ng of DNA template containing CAT coding region, 200 µM dNTP and 1 unit Taq DNA polymerase.

PCR was performed as follows: denaturation at 94 °C for 30 seconds, annealing for 45 seconds at 55 °C and extension for 2 minutes at 72 °C for 25 cycles. The PCR product was analyzed by electrophoresis in 1% agarose gel and purified using a commercial PCR cleaning kit. A second PCR reaction was carried out using the product from the first PCR as template. The reaction mix also contained 5 ng of DNA fragment (800 bp) comprising a modified promoter sequence from human cytomegalovirus (Gene Thearpy Systems, San Diego, CA), 5 ng of DNA fragment (200 bp) SV40 transcription terminator region, and 400 ng of primers CMV154 and SV40-2. The PCR was performed under similar conditions as above except the annealing temperature was raised to 60 °C and the extension time was extended to 3 minutes. The resulting PCR product was transcriptionally active and was used directly for transfection of cells *in vitro* or tisuues *in vivo*.

EXAMPLE 2

Cloning of transcriptionally active PCR fragment encoding Chloramphenicol acetyltransferase (CAT)

The PCR fragment of Example 1 was cloned by mixing 0.5 µg of the final PCR product with 0.1 µg of plasmid pCMVm-SV40-T that was linearized and had sequences identical to the sequences flanking the CAT gene in the PCR fragment. The mixed PCR product and linear vector were transformed into *E. coli* JC8679 through electroporation

followed by incubation in SOC medium at 37 °C for 1 hour and plating on a LB/agar plate containing 100 μg/ml Kanamycin for selection over night at 37 °C. Colonies were selected and miniprep DNA was isolated for further analysis and insertion of the PCR product into the vector.

EXAMPLE 3

Forced cloning using a suicide gene

A plasmid is constructed in such that the toxic gene GATA-1 is under the control of tac (IPTG-inducible) promoter. The GATA-1-expressing unit is then flanked by TAP promoter (modified CMV IE promoter/intron, 800 bp) and TAP terminator (SV40 transcription terminator, 200 bp) sequences. A Transcriptionally active PCR fragment encoding CAT gene is generated using the same promoter and terminator elements. 2 μg of such TAP fragments is transformed into competent bacteria cells that contains the GATA-1/TAC plasmid and prepared in the absence of IPTG. After transformation, bacteria are plated on a LB plate containing 10 ng/ml IPTG. Only the cells bearing the plasmid in which the TAC/ATA-1 is replaced by the TAP fragment encoding gene of interest are able to grow.

While particular embodiments of the invention have been described in detail, it will be apparent to those skilled in the art that these embodiments are exemplary rather than limiting, and the true scope of the invention is that defined in the following claims.